



Original Contribution

## Postischemic hyperoxia reduces hippocampal pyruvate dehydrogenase activity

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### Abstract

The pyruvate dehydrogenase complex (PDHC) is a mitochondrial matrix enzyme that catalyzes the oxidative decarboxylation of pyruvate and represents the sole bridge between anaerobic and aerobic cerebral energy metabolism. Previous studies demonstrating loss of PDHC enzyme activity and immunoreactivity during reperfusion after cerebral ischemia suggest that oxidative modifications are involved. This study tested the hypothesis that hyperoxic reperfusion exacerbates loss of PDHC enzyme activity, possibly due to tyrosine nitration or S-nitrosation. We used a clinically relevant canine ventricular fibrillation cardiac arrest model in which, after resuscitation and ventilation on either 100% O<sub>2</sub> (hyperoxic) or 21–30% O<sub>2</sub> (normoxic), animals were sacrificed at 2 h reperfusion and the brains removed for enzyme activity and immunoreactivity measurements. Animals resuscitated under hyperoxic conditions exhibited decreased PDHC activity and elevated 3-nitrotyrosine immunoreactivity in the hippocampus but not the cortex, compared to nonischemic controls. These measures were unchanged in normoxic animals. In vitro exposure of purified PDHC to peroxynitrite resulted in a dose-dependent loss of activity and increased nitrotyrosine immunoreactivity. These results support the hypothesis that oxidative stress contributes to loss of hippocampal PDHC activity during cerebral ischemia and reperfusion and suggest that PDHC is a target of peroxynitrite.

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Global cerebral ischemia and reperfusion cause severe neurochemical alterations, including oxidative modifications to lipids, proteins, and DNA. These and other covalent modifications can impair enzyme activities, including those necessary for energy metabolism. Alterations in these enzyme activities can limit postischemic aerobic metabolism and cellular ATP regeneration, thereby contributing to the pathophysiology of delayed neuronal cell death [1–4]. One enzyme known to be targeted and inactivated by reactive oxygen species is the pyruvate dehydrogenase complex (PDHC) [5]. Effects of ischemia/reperfusion on the PDHC can be particularly devastating because this enzyme forms the bridge between glycolysis

and the Krebs cycle and can be the rate-limiting step in aerobic cerebral energy metabolism.

The PDHC is located exclusively in the mitochondrial matrix and catalyzes the oxidative decarboxylation of pyruvate to form NADH and acetyl coenzyme A—the primary form of carbon that enters the Krebs cycle in the adult brain. Several laboratories have shown that PDHC enzyme activity is lost during cerebral ischemia/reperfusion [6–8]. PDHC immunoreactivity is also reduced within a few hours of reperfusion and this loss is most pronounced within neurons that are selectively vulnerable to subsequent cell death [6]. One proposed mechanism of reperfusion-dependent enzyme inactivation involves the attack of free radicals on the enzyme complex. Purified PDHC is highly sensitive to inactivation by exposure to hydroxyl radicals [5]. It is not known, however, whether inactivation occurs after exposure to other forms of oxidative stress, e.g., nitric oxide and peroxynitrite, that are generated at

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abnormally high levels shown to promote postischemic neuronal injury during ischemic reperfusion [9,10].

Peroxynitrite (ONOO<sup>-</sup>) is a reactive nitrogen species known to increase after ischemia/reperfusion [11,12]. Peroxynitrite is generated by the nonenzymatic reaction between superoxide and nitric oxide and modifies proteins via several different mechanisms, including protein S-nitrosation and nitration of tyrosine residues. 3-Nitrotyrosine (NT) serves as a footprint of peroxynitrite formation, occurs within neurons during ischemic reperfusion [13], and is exacerbated by the presence of high inspired O<sub>2</sub> [14]. Considering our evidence that hyperoxic resuscitation after cardiac arrest worsens neurologic outcome, increases lipid oxidation, and elevates brain lactate levels [15], we tested the hypothesis that loss of PDHC enzyme activity is also promoted by hyperoxic, compared to normoxic, reperfusion. The results of this study support this hypothesis and also provide evidence that peroxynitrite mediates oxidative modification and inactivation of this enzyme during ischemic reperfusion.

## Materials and experimental methods

### Materials

All chemicals and reagents were purchased from Sigma–Aldrich unless otherwise stated.

### Canine cardiac arrest model

Animal experimentation was performed according to the guidelines of the Institutional Animal Use and Care Committee of the University of Maryland, Baltimore. The animal model used for these studies has been used extensively by us and others to study global cerebral ischemia and reperfusion [15–20]. Adult purebred female beagles weighing 10–15 kg were anesthetized initially with an intravenous injection of veterinary thiopental (8–12 mg/kg). Prolonged anesthesia was then induced with an infusion of  $\alpha$ -chloralose (75 mg/kg). After establishment of appropriate anesthesia, animals were intubated endotracheally and subjected to controlled ventilation on room air (21% O<sub>2</sub>). Cut-down catheters were placed in the left femoral artery and vein. The arterial catheter was attached to a transducer for continuous monitoring of arterial pressure. The venous catheter was advanced to the level of the inferior vena cava for the purpose of resuscitative drug delivery. An intravenous infusion of normal saline (3 ml/kg/h) was started at this point and continued for the remainder of the experiment. A left lateral thoracotomy was performed through the fourth intercostal space. The pericardium was incised and reflected. Core temperature was monitored continuously and maintained between 37 and 38°C with a heating blanket and heat lamps.

After surgical preparation, cardiac arrest was induced with an electrical train of currents generated by a Grass stimulator applied directly to the epicardium. The presence of cardiac arrest was verified by EKG rhythm consistent with ventricular fibrillation (VF) in the presence of systolic arterial pressure (SAP) of <20 mm Hg. At the initiation of VF, artificial

ventilation was discontinued. VF was allowed to continue without treatment for 10 min. At the end of 10 min of VF, ventilation was resumed (respiratory rate 28) simultaneous with the beginning of manual open chest cardiopulmonary resuscitation (OCCPR) at a rate of 56/min. OCCPR was standardized such that SAP remained between 90 and 120 mm Hg throughout. Epinephrine (0.2 ml/kg of 1/10,000 solution) and sodium bicarbonate (1 mEq/kg) were injected at the beginning of resuscitation through the femoral central venous catheter. OCCPR was continued for 3 min at which point internal defibrillation was performed at 5 J. Arterial blood gas determinations were made immediately after resuscitation and at 10, 30, 60, and 90 min thereafter.

Animals were assigned to one of three resuscitative protocols. For the hyperoxic protocol, resuscitation was performed with 100% ventilatory O<sub>2</sub> during the OCCPR and for 1 h thereafter. During the next hour the ventilator settings were adjusted to maintain arterial pO<sub>2</sub> >80 and <120 mm Hg. For normoxic conditions, dogs were resuscitated with room air (21% O<sub>2</sub>) and then inspired O<sub>2</sub> was rapidly adjusted, if necessary, between 21 and 30% to maintain pO<sub>2</sub> >80 and <120 mm Hg. For the sham operation, dogs were ventilated on room air and underwent all anesthetic and surgical procedures but did not undergo cardiac arrest. pCO<sub>2</sub> was maintained between 25 and 35 mm Hg in all animals for the duration of the experiment. Exclusion criteria included temperature <37°C, SAP <60 at any time after resuscitation, or inability to maintain pO<sub>2</sub> or pCO<sub>2</sub> within stated limits.

### Postischemic tissue processing

After cardiac arrest/resuscitation and 2 h reperfusion, a 3-cm-long/2-cm-wide/1-cm-thick portion of the frontal cortex from one hemisphere was removed and placed into ice-cold mannitol–sucrose (MS) isolation buffer that contained 225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mg/ml BSA (fatty acid free), 1 mM EGTA, pH 7.4. The entire opposite hemisphere was also removed and placed into ice-cold MS buffer and the entire hippocampus was excised in a cold room within 3 min after the hemisphere was removed. The cortex and hippocampal samples were then separately homogenized using a Dounce tissue homogenizer (Fisher, Hampton, NH, USA) and protein concentration was determined according to the method of Lowry et al. [78]. An aliquot of homogenate was immersed in liquid nitrogen and stored at –80°C until further processing for enzyme activity and immunoreactivity.

### PDHC enzyme activity

The brain homogenate stored at –80°C was removed from the freezer and thawed on ice. The homogenate was diluted to a concentration of 2–6 mg protein/ml in ice-cold buffer containing 50 mM potassium phosphate (pH 7.8), 1 mM EDTA, and 0.1% (wt/vol) Triton X-100 and placed through two freeze–thaw cycles. In some experiments, samples were freeze–thawed in the same medium supplemented with 10 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, and 0.004 units of pyruvate dehydrogenase phosphatase

to ensure complete protein dephosphorylation and therefore maximal enzyme activity. PDHC activity was measured using a method adapted from one previously published in which the reduction of NAD to NADH is enzymatically coupled to the reduction of a fluorescent dye [22]. In our procedure, the enzyme diaphorase was used to reduce resazurin to resorufin, which is fluorometrically measured at 585-nm excitation and 550-nm emission wavelengths. The freeze-thawed homogenate was added to a 50 mM potassium phosphate buffer (pH 7.8, 37°C) that contained 0.5 mM thiamine pyrophosphate (TPP), 5 mM MgCl<sub>2</sub>, 0.2 mM coenzyme A, 5 mM NAD, 2 U phosphotransacetylase, 0.3 U diaphorase, and 1 mM resazurin. In addition, 0.5 mM oxalate, an inhibitor of lactate dehydrogenase, was added to minimize interference from this reaction [23]. Background fluorescence was recorded for 30 s at which time the reaction was initiated by adding 5 mM pyruvate. Initial PDHC activity was determined by measuring the slope of fluorescence increase during the first 2 min after addition of pyruvate and subtracting out the background change in fluorescence (<10% of the rate plus pyruvate).

#### *Immunoprecipitation of nitrated PDHC*

Previously frozen canine hippocampal homogenates were removed from the freezer and placed on ice. Samples were diluted to 1 mg/ml and a 3-nitrotyrosine affinity sorbent cocktail (Cayman, Ann Arbor, MI, USA) was used to capture all nitrated proteins according to the manufacturer's specifications. The captured proteins were then prepared for immunoblot analysis of PDHC immunoreactivity. The immunoreactivity of the light-chain IgG band was used to normalize the data.

#### *Effect of peroxynitrite on purified PDHC enzyme activity*

We incubated purified porcine PDHC (Sigma) in the presence of various concentrations of purified peroxynitrite or the peroxynitrite-generating system 3-morpholininosydnonimine (SIN-1) (Molecular Probes, Eugene, OR, USA) to determine if the enzyme is a target of oxidative stress *in vitro*. The enzyme was diluted to 2 mg/ml in 50 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.8) containing 5 mM MgCl<sub>2</sub>. One hundred microliters of the diluted enzyme was added to a final volume of 2 ml phosphate buffer and incubated at 37°C in the absence or presence of 0.03–7.2 mM SIN-1 for durations of 10–60 min, with continuous stirring. Where indicated, 10 mM DMSO, 60 U Cu/Zn superoxide dismutase, or 10 mM dithiothreitol (DTT) was also included during the preincubation period. After the incubation, 0.5 mM TPP, 0.2 mM coenzyme A, and 5 mM NAD were added to the cuvette. In a subset of experiments, one or more of these cofactors were present during the preincubation. A background spectrophotometric reading was obtained for approximately 30 s at which time the reaction was initiated with the addition of 5 mM pyruvate. Enzyme activity was determined by spectroscopy at 340 nm.

To ensure that the effects of SIN-1 on enzyme activity were attributed to peroxynitrite, and not nitric oxide or superoxide

separately, we repeated a subset of the experiments described above with incubation in the presence or absence of prepared ONOO<sup>-</sup> (Upstate, Lake Placid, NY, USA). Concentrations of ONOO<sup>-</sup> used for experimentation are based upon information provided by the supplier and may vary due to slow decomposition during storage. Thus, concentrations indicated are meant to serve as only an estimate. After the enzyme activity assays, samples were placed in cryogenic vials, immersed in liquid nitrogen, and stored at -80°C until immunoblot analysis or ELISA was performed.

#### *Immunoblot analysis and ELISA*

To determine PDHC and 3-nitrotyrosine immunoreactivity, samples were removed from the freezer and thawed on ice. In preparation for immunoblot analysis, 50 µg canine cortical and hippocampal homogenate protein samples were diluted in SDS sample buffer (Invitrogen, Carlsbad, CA, USA) plus dithiothreitol. Samples were heated to 80°C, loaded on a 4–12% Bis-Tris gel (Invitrogen), separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Immunodetection for 3-nitrotyrosine (rabbit polyclonal; Upstate) or PDHC (mouse monoclonal antibody cocktail; Mitosciences, Eugene, OR, USA) was performed. The Upstate antibody was used at a primary antibody dilution of 1:1000, followed by secondary antibody at a 1:10,000 dilution. The PDHC antibody cocktail was used at a 1:500 primary antibody dilution, followed by a 1:10,000 dilution of secondary antibody. Nitrotyrosine quantification was also implemented according to the manufacturer's instructions using a NT ELISA kit (Cell Sciences, Canton, MA, USA).

#### *Statistical analysis*

One-way ANOVA followed by Tukey post hoc analysis was implemented for statistical analysis of experimental groups resuscitated under different oxygen tensions after cardiac arrest. Student's *t* test was implemented to determine significance of *in vitro* experimental groups and the arterial *p*O<sub>2</sub> values for the two reperfusion animal groups. Data expressed as percentage of control (PDHC activity) were log transformed before analysis, which tended to produce a more Gaussian distribution. Regression analysis (test for trend) was performed to determine if [SIN-1] significantly affected PDHC activity and/or NT immunoreactivity. A value of *p* < 0.05 was considered significant.

## **Results**

#### *Canine cardiac arrest and resuscitation*

Whereas there were no significant differences in baseline physiologic parameters, including pH, temperature, *p*O<sub>2</sub>, and *p*CO<sub>2</sub> between animal groups, the *p*O<sub>2</sub> at 30 min reperfusion for hyperoxic animals (467.6 ± 29.7 mm Hg, SE, *n* = 8) was significantly greater (*p* < 0.001) than the *p*O<sub>2</sub> of normoxic resuscitated animals (89.2 ± 4.3 mm Hg, SE, *n* = 8).

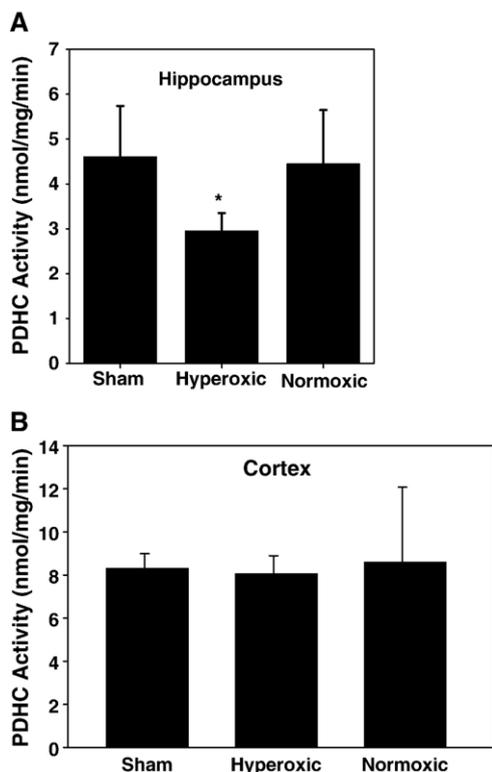


Fig. 1. Selective inhibition of hippocampal pyruvate dehydrogenase complex enzyme activity by hyperoxic resuscitation after cardiac arrest. PDHC maximal enzyme activity was measured spectrofluorometrically using tissue homogenates obtained from samples of the (A) hippocampus and (B) frontal cortex of sham-operated (nonischemic) dogs or dogs at 2 h after 10 min cardiac arrest with resuscitation using either hyperoxic or normoxic ventilation. Values represent the means  $\pm$  SE for  $n = 5$  animals per group. \*Significantly different from sham-operated control and normoxic-resuscitated animals groups; one-way ANOVA with Tukey post hoc analysis;  $p < 0.05$ .

#### Postischemic hippocampal and frontal cortex PDHC enzyme activity

The values for brain tissue maximal PDHC specific activity obtained in these experiments are within the range of previously published results for canine PDHC activity [5]. After 10 min global ischemia followed by 2 h reperfusion, animals that were ventilated under hyperoxic conditions exhibited approximately 37.5% less hippocampal homogenate PDHC activity ( $p < 0.05$  one-way ANOVA; Tukey post hoc analysis), compared to sham-operated control animals or those resuscitated under normoxic conditions (Fig. 1A). Comparisons were made of enzyme activity in some samples that were thawed in the absence and presence of PDHC phosphatase plus added  $MgCl_2$  and  $CaCl_2$  to verify that the enzyme was completely dephosphorylated and therefore maximally active (see Materials and experimental methods). No differences in activity were observed when samples were thawed in the absence or presence of PDHC phosphatase (not shown).

In general, neurons within the hippocampus are selectively vulnerable to delayed cell death after global cerebral ischemia/reperfusion compared to neurons in the frontal cortex [24,25]. Postulated reasons for selective vulnerability include differ-

ences in susceptibility to oxidative stress, excitotoxic receptor activation, apoptotic mechanisms, and metabolic failure [26–29]. To determine if early, prelethal loss of PDHC activity relates to selective vulnerability, we also measured PDHC activity in homogenates of frontal cortex. In contrast to the loss of enzyme activity observed in the hyperoxic hippocampal samples, there was no significant change in cortical PDHC activity in samples from either the hyperoxic or the normoxic animal groups (Fig. 1B).

#### Postischemic nitrotyrosine measurements

Previous HPLC measurements of oxidized fatty acyl groups present in the brains of hyperoxic and normoxic resuscitated animals indicated that hyperoxic ventilation exacerbates postischemic oxidative modification of lipids [15]. To determine if hyperoxia also promotes protein oxidation, we measured levels of 3-nitrotyrosine immunoreactivity in the hippocampus and cortex of nonischemic animals and at 2 h reperfusion after hyperoxic or normoxic resuscitation. In the hippocampus of animals resuscitated under hyperoxic conditions, an approximate 26% increase in 3-nitrotyrosine immunoreactivity was detected by ELISA, compared to sham-operated control animals and those resuscitated under normoxic conditions ( $p < 0.05$ , one-way ANOVA, Tukey post hoc analysis) (Fig. 2A). This difference was not observed in the

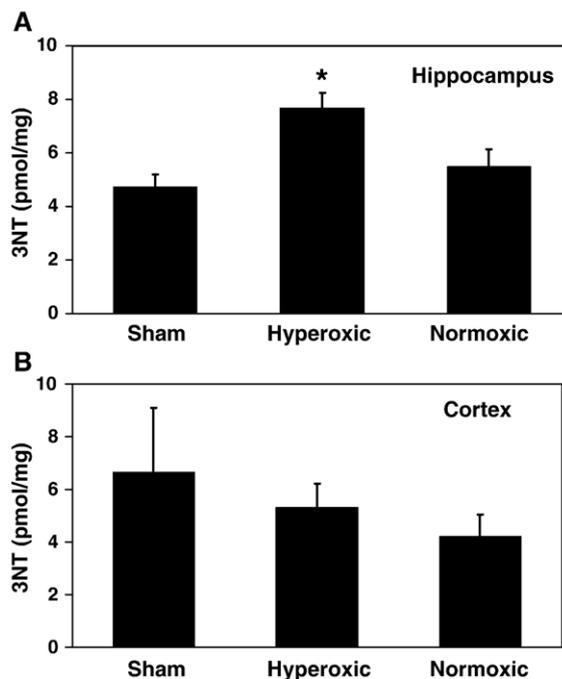


Fig. 2. Selective elevation of hippocampal 3-nitrotyrosine immunoreactivity by hyperoxic resuscitation after cardiac arrest. 3-Nitrotyrosine immunoreactivity was measured by ELISA using tissue homogenates obtained from samples of the (A) hippocampus and (B) frontal cortex of sham-operated (nonischemic) dogs or dogs at 2 h after 10 min cardiac arrest with resuscitation using either hyperoxic or normoxic ventilation. Values represent the means  $\pm$  SE for  $n = 5$  animals per group. \*Significantly different from sham-operated control and normoxic-resuscitated animals groups; one-way ANOVA with Tukey post hoc analysis;  $p < 0.05$ .

cortex of these animals, consistent with the preservation of PDHC activity in this brain region (Fig. 2B).

Whereas correlative relationships exist between loss of PDHC enzymatic activity and 3-nitrotyrosine immunoreactivity, with regard to both brain region and resuscitation protocol, we performed immunoprecipitation experiments to provide more direct evidence that PDHC is subject to tyrosine nitration during reperfusion. Figs. 3A and 3C are images generated from a representative immunoblot using a cocktail of monoclonal antibodies to PDHC subunits applied to proteins from hippocampal homogenates precipitated with an antibody to 3-nitrotyrosine. PDHC E2 subunit immunoreactivity present in samples from the hyperoxic-resuscitated animals was significantly greater than that of normoxic-resuscitated animals or sham-operated controls (Fig. 3B). A trend toward increased levels of other subunits was also observed (Fig. 3C). We therefore conclude that PDHC contains tyrosine residues that are nitrated during cerebral ischemia and hyperoxic reperfusion.

#### Inhibition of purified PDHC enzyme activity by SIN-1

Based on the relationship between nitrotyrosine immunoreactivity and loss of PDHC enzyme activity between brain

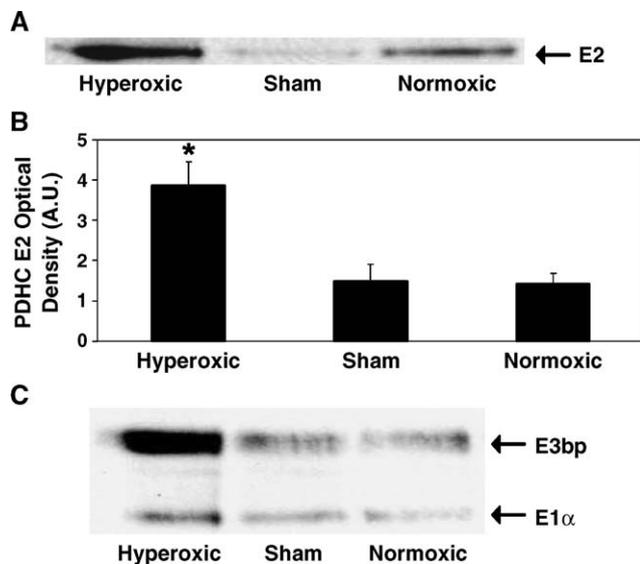


Fig. 3. Pyruvate dehydrogenase complex subunit immunoreactivity in anti-nitrotyrosine-immunoprecipitated hippocampal and cortical homogenate proteins. A nitrotyrosine affinity sorbent was used to capture all nitrated proteins in the hippocampal homogenate samples. Western blot analysis was then performed using a monoclonal antibody cocktail to several PDHC subunits. This antibody reacts the strongest with the E2 subunit of the enzyme although immunoreactivity with several other subunits was also observed. (A) Representative immunoblot comparing PDHC E2 immunoreactivity of a sample from a sham-operated (nonischemic) animal and from animals at 2 h reperfusion using either normoxic or hyperoxic resuscitation. (B) Densitometric analysis of PDHC E2 immunoreactivity. Values are reported in arbitrary units of optical density and are normalized for loading differences by optical density of the IgG light chain. Values represent the means  $\pm$  SE for  $n = 5$  animals per group. \*Significantly different from sham-operated control and normoxic-resuscitated animal groups; one-way ANOVA with Tukey post hoc analysis;  $p < 0.05$ . (C) Representative immunoblot depicting the immunoreactivity of other PDHC subunits.

regions and resuscitative animal groups, we hypothesized that purified PDHC is sensitive to inactivation by peroxynitrite. This hypothesis was initially tested by exposing purified porcine PDHC to SIN-1, an agent that produces both superoxide and nitric oxide, which react with each other forming peroxynitrite. After a 20-min incubation in the absence of enzyme cofactors, maximal PDHC activity was reduced by exposure to SIN-1 in a dose-dependent manner ( $K_i \approx 50 \mu\text{M}$ ) (Fig. 4C, solid line). After the incubations, the solution containing purified PDHC was also tested for 3-nitrotyrosine immunoreactivity. As expected, exposure to SIN-1 resulted in a dose-dependent increase in PDHC 3-nitrotyrosine immunoreactivity (Figs. 4A and 4C). PDHC immunoreactivity was not, however, lost after incubation with SIN-1 (Fig. 4B). A similar reciprocal relationship between 3-nitrotyrosine immunoreactivity and PDHC enzymatic activity was observed with various periods of exposure to a constant concentration of SIN-1 (Fig. 5).

Previous experiments demonstrated that the presence of certain PDHC substrates and cofactors protects against loss of enzymatic activity caused by exposure to the hydroxyl radical [5]. We therefore tested the ability of these molecules to protect against the loss of enzymatic activity observed after incubation with SIN-1. Significant, albeit incomplete, protection against loss of enzyme activity was observed in the presence of either pyruvate or coenzyme A (Table 1). When the enzymatic reaction was started with the addition of coenzyme A and all other substrates plus TPP were present during preincubation with SIN-1, approximately 10 times higher SIN-1 concentrations were needed to inhibit enzyme activity compared to those effective in the absence of substrates (Fig. 4D). The reciprocal relationship between 3-nitrotyrosine immunoreactivity and PDHC enzymatic activity was observed under these conditions, as it was in the absence of substrates during the SIN-1 exposure.

Experiments were also performed to determine the relative contribution of tyrosine nitration and S-nitrosation to the inactivation of PDHC by peroxynitrite. PDHC was incubated in the presence of SIN-1 and in the absence and presence of DTT, a thioreductant, which protects against S-nitrosation of cysteines. Compared to PDHC activity in the absence of SIN-1 exposure, we observed a significant decrease in enzyme activity ( $40.2 \pm 3.2\%$ ;  $p < 0.05$ , Student's  $t$  test) in the presence of  $40 \mu\text{M}$  SIN-1. In the presence of  $40 \mu\text{M}$  SIN-1 +  $10 \text{ mM}$  DTT, enzyme activity was also reduced ( $28.9 \pm 3.0\%$ ;  $p < 0.05$ ) (Fig. 6). Thus whereas protection against S-nitrosation salvaged some of the enzymatic activity, most of the SIN-1-induced inhibition was insensitive to DTT and was therefore likely due to tyrosine nitration.

Experiments were also performed to confirm that inhibition of PDHC enzymatic activity by SIN-1 is caused by peroxynitrite rather than the substrates generated by SIN-1 (superoxide and nitric oxide) or the hydroxyl radical, which can be generated during the degradation of peroxynitrite. The presence of superoxide dismutase during the 20-min incubation of PDHC with SIN-1 protected against inhibition of enzyme activity (Fig. 6A). As superoxide dismutase eliminates superoxide and therefore peroxynitrite but does not affect nitric oxide formation, nitric oxide, per se, is not responsible for enzymatic

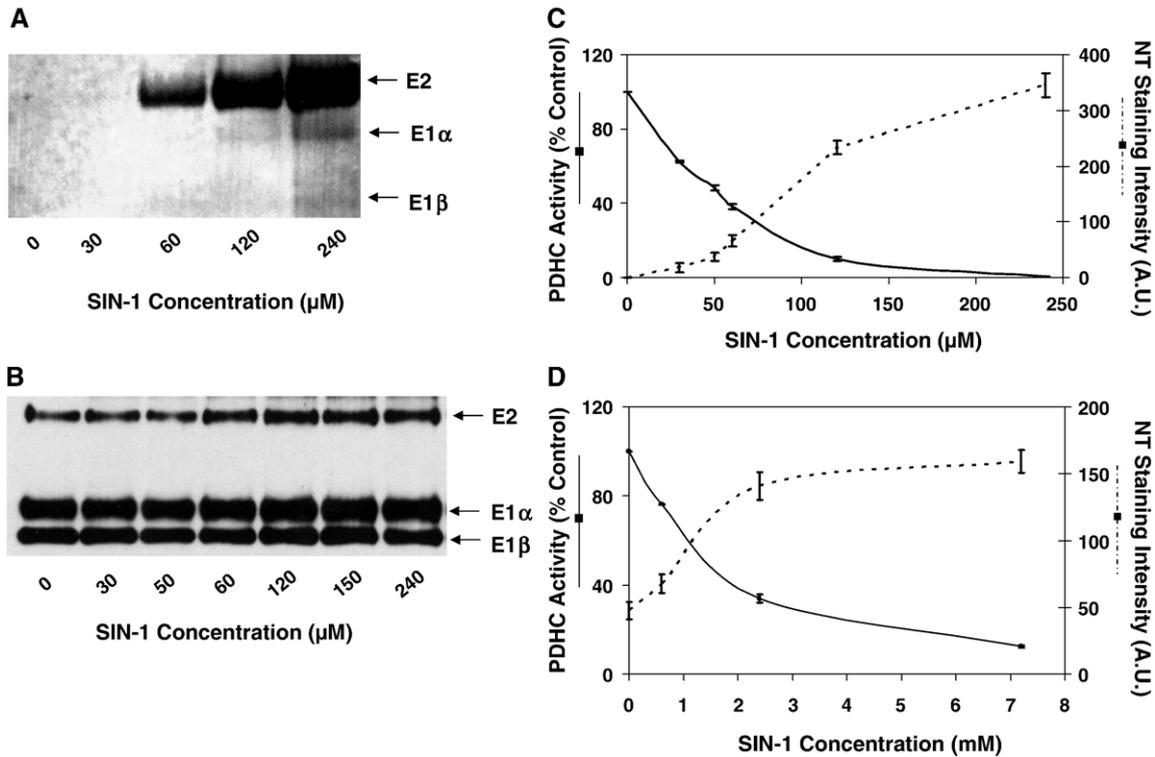


Fig. 4. Effects of SIN-1 on purified pyruvate dehydrogenase complex enzyme activity, immunoreactivity and protein tyrosine nitration. Purified PDHC (200 μg) was incubated in the presence of increasing concentrations of the peroxynitrite-generating system 3-morpholinosydnonimine (SIN-1) for 20 min. After the incubation period, the required substrates and cofactors were added to initiate enzyme activity, which was measured spectrophotometrically. (A) 3-Nitrotyrosine immunoblot. (B) PDHC immunoblot. (C) Quantification of PDHC enzyme activity and 3-nitrotyrosine (NT) immunoreactivity after exposure of purified enzyme to SIN-1 in the absence of required substrates and cofactors. (D) Quantification of PDHC enzyme activity and NT immunoreactivity after exposure of purified enzyme to SIN-1 in the presence of all enzyme substrates and cofactors except coenzyme A, which was used to start the enzyme reaction. PDHC activity is reported as % of control activity measured after preincubation in the absence of SIN-1. A regression analysis (test for trend) was performed for the data located in (C) and (D). NT optical density is reported in arbitrary units (A.U.). Values represent the means ± SE for n = 6 experiments per [SIN-1].

inhibition by SIN-1. Attempts to eliminate nitric oxide by the inclusion of hemoglobin were unsuccessful due to interference with the spectrophotometric enzyme assay that uses absorbance of light at 340 nm. To further ensure that the observed effects of SIN-1 on PDHC are due to ONOO<sup>-</sup> production, we tested for

the effects of commercially prepared ONOO<sup>-</sup> on PDHC enzyme activity and observed a dose-dependent inhibition (Fig. 6B). When using either SIN-1 or prepared ONOO<sup>-</sup>, it is possible that a peroxynitrite degradation product, particularly the hydroxyl radical, could be responsible for enzyme inhibition. This possibility was addressed by testing the effects of the hydroxyl radical scavenger DMSO on the inhibitory effect of SIN-1 on PDHC activity. The presence of 10 mM DMSO during the preincubation period had no effect on enzymatic activity, further pointing toward ONOO<sup>-</sup> as the species responsible for inactivation of PDHC (Fig. 6A).

**Discussion**

The most important new observations reported in this study are that hyperoxic resuscitation after cardiac arrest reduces hippocampal PDHC enzyme activity and elevates 3-nitrotyrosine immunoreactivity compared to values obtained with sham-operated control animals or those resuscitated using normal arterial O<sub>2</sub> levels. The finding that hippocampal 3-nitrotyrosine immunoreactivity measured by ELISA is elevated after hyperoxic but not normoxic resuscitation is consistent with results we obtained with 3-nitrotyrosine immunohistochemistry [14] and with the observation that hyperoxic resuscitation increases brain oxidized free fatty acyl groups in this model

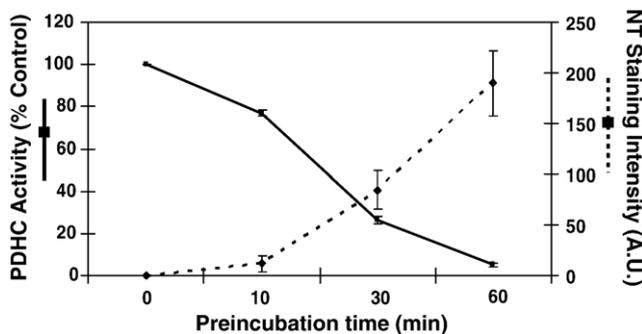


Fig. 5. Time-dependent inhibition of pyruvate dehydrogenase complex enzyme activity and 3-nitrotyrosine immunoreactivity by SIN-1. Purified PDHC (200 μg) was incubated in 50 μM SIN-1 for 0–60 min in the absence of enzyme substrates and cofactors before evaluation of nitrotyrosine (NT) immunoreactivity and enzyme activity. PDHC enzyme activity is reported as % of control activity measured after preincubation in the absence of SIN-1. NT optical density is reported in arbitrary units (A.U.). Values represent the mean ± SE for n = 4 experiments per incubation time.

Table 1  
Protection against SIN-1 inhibition of pyruvate dehydrogenase complex enzyme activity by the presence of enzyme substrates

Substrate/cofactor present during SIN-1 exposure	PDHC activity (% control)
None	22.0 ± 1.7
Pyruvate	62.5 ± 4.2 *
NAD	28.7 ± 3.1
TPP	10.9 ± 1.0
Coenzyme A	35.2 ± 3.5 **

Enzyme activity was measured after a 20-min preincubation with 100 μM SIN-1 and is expressed as % control activity present after incubation in the absence of SIN-1. Values represent the means ± SE for *n* = 4 independent experiments.

\* Significantly different from incubation with SIN-1 in the absence of substrates and cofactors (*p* < 0.001).

\*\* Significantly different at *p* < 0.05.

[15]. More importantly, the exacerbation of tyrosine nitration during early reperfusion by hyperoxic resuscitation is associated with worse neurologic outcome [15] and greater hippocampal cell death compared to that observed after normoxic resuscitation [14]. These relationships add to the body of evidence that protein tyrosine nitration contributes to a wide range of neuropathologies, including traumatic brain injury [30,31], focal ischemia [32], global ischemia [33,34], and neurodegenerative diseases [35,36].

The chemical basis for increased tyrosine nitration in the hippocampus after hyperoxic resuscitation is most likely linked to the stimulation of peroxynitrite formation by increased production of superoxide and (or) nitric oxide, the two substrates required for peroxynitrite formation. Whereas prolonged hyperoxia can stimulate the expression of inducible nitric oxide synthase [37], increased levels of this protein are not likely to occur within the 2-h reperfusion period used in our measurements. Elevated brain tissue O<sub>2</sub> tension could, however, directly increase the rate of existing redox protein-mediated production of both nitric oxide and superoxide. For example, several studies indicate that mitochondrial superoxide production is directly related to [O<sub>2</sub>] (between normal *p*O<sub>2</sub> up to 100% O<sub>2</sub> saturation) [38,39]. Although reports indicate that severe hypoxia can also stimulate superoxide production [40–42], promotion of superoxide generation by high brain tissue *p*O<sub>2</sub> is a more likely explanation for the increased 3-nitrotyrosine immunoreactivity in the hyperoxic-resuscitated animals. In addition, the *K<sub>m</sub>* of O<sub>2</sub> for nitric oxide synthases has been reported to be as low as micromolar or as high as millimolar concentrations, suggesting that elevated tissue O<sub>2</sub> tension could also directly stimulate nitric oxide production [37,43,44]. As microdialysis measurements indicate that total end products of nitric oxide generation are elevated within 20–60 min of reperfusion after transient global cerebral ischemia [45], it follows that hyperoxic resuscitation should increase markers of peroxynitrite production, e.g., 3-nitrotyrosine immunoreactivity.

Although many cellular proteins are vulnerable to tyrosine nitration, several factors may predispose PDHC toward tyrosine nitration and inactivation in situ within the mitochondrial environment. When mitochondria are exposed to nitrating

agents, the mitochondrial matrix, where PDHC is located, exhibits the greatest nitration compared to other mitochondrial fractions (i.e., outer membrane, inner membrane, and intermembrane space) [46]. PDHC is closely associated with Complex I of the electron transport chain [47] where much of the endogenous superoxide production is thought to occur. PDHC and α-ketoglutarate dehydrogenase complex may also be important sources of superoxide and, consequently, peroxynitrite [48,49]. The free radical pathways leading to nitration are initiated secondary to the reaction of peroxynitrite with carbon dioxide [50] and PDHC is one of the main sources of carbon dioxide within the mitochondria [51]. The relative sensitivity of PDHC to tyrosine nitration after cerebral ischemia and reperfusion is at this juncture unknown. A proteomic analysis, such as those that have identified PDHC as a highly nitrated protein in other paradigms [46,52,53] would likely resolve this question.

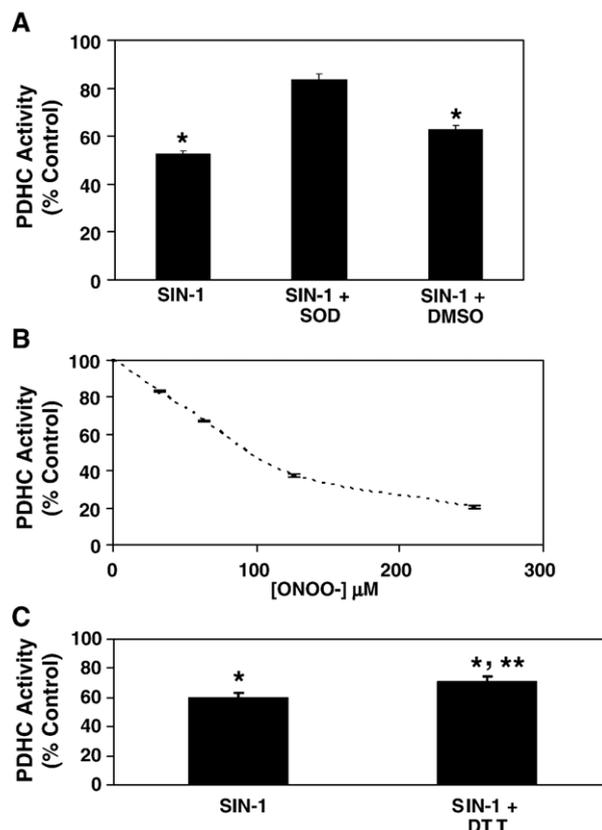


Fig. 6. Confirmation that peroxynitrite mediates inhibition of pyruvate dehydrogenase by SIN-1. (A and C) Purified PDHC (200 μg) was incubated for 20 min with or without 50 μM 3-morpholinisydnonimine (SIN-1). In (A), 60 units of Cu/Zn superoxide dismutase (SOD) or 10 mM DMSO was present during the incubation with SIN-1, as indicated. In (C), the enzyme was incubated in the absence or presence of 10 mM dithiothreitol (DTT). (B) PDHC was incubated in the same buffer as in (A) and (C) except that SIN-1 was replaced with various concentrations of prepared peroxynitrite. Values are reported as % of control activity measured after preincubation in the absence of SIN-1 or peroxynitrite and represent the means ± SE for *n* = 6 separate determinations. \*Significantly different from control activity, and \*\*significantly different from activity in the presence of SIN-1 and in the absence of DTT, based on one-way ANOVA, Dunnett post hoc analysis; *p* < 0.05.

Several other laboratories have also reported postischemic impairment of PDHC activity. Decreased activity of the PDHC in the dorsolateral striatum differentiates this region from ischemia-resistant areas during early recirculation in a rat model of transient ischemia, providing additional evidence for a relationship between PDHC inactivation and selective neuronal cell death [8]. Whereas in our present study we observed a significant loss of PDHC activity in the hippocampus but not the frontal cortex (Fig. 1), results obtained previously with a different reperfusion protocol demonstrated loss of activity in the cortex [5]. Depending on the experimental conditions, reperfusion-dependent inhibition of PDHC may therefore occur in several brain regions but is greater in the hippocampus compared to the cortex using the hyperoxic resuscitation protocol employed in this study. Our findings that hyperoxic reperfusion *in vivo* and exposure to peroxynitrite *in vitro* inhibit PDHC activity are likely attributed to one or more of the following causes: (1) phosphatase activity is inhibited, maintaining PDHC in the inactive state; (2) PDH kinase activity is enhanced, forming phosphorylated (inactive) PDHC; (3) dephosphorylation of the enzyme is prohibited by oxidative modification of the enzyme; or (4) maximal activity of dephosphorylated (active) PDHC is lost. Addition of exogenous phosphatase in the presence of  $Mg^{2+}$  and  $Ca^{2+}$  did not increase enzyme activity, indicating that PDH phosphatase activity is not impaired in these animals. Similarly, because the assay conditions promote maintenance of the enzyme in its dephosphorylated (active) state and the presence of dichloroacetate, an inhibitor of PDHC phosphorylation, had no effect on PDHC activities (not shown), impaired kinase activity is not responsible for the inhibition of activity after hyperoxic reperfusion. It is nevertheless possible that the phosphorylated enzyme is targeted by peroxynitrite and modified such that it is no longer susceptible to dephosphorylation. However, given our *in vitro* experiments that show that “active” purified PDHC is targeted and inactivated by peroxynitrite, the most likely mechanism of enzyme inhibition is that PDHC in its active, dephosphorylated form is susceptible to attack and subsequent inactivation by peroxynitrite. In addition, loss of PDHC activity after cerebral ischemia and reperfusion in rat arterial occlusion models is the result of impaired maximal activity rather than covalent inhibition caused by increased protein phosphorylation [21,54].

The results obtained *in vivo* suggesting that peroxynitrite mediates postischemic PDHC enzyme inhibition are supported by *in vitro* experimentation in which incubation of purified PDHC with SIN-1 is associated with decreased PDHC activity and increased 3-nitrotyrosine immunoreactivity (Figs. 4–6). Tyrosine nitration is involved in many postischemic modifications [55–57]. Examples of proteins susceptible to inactivation by tyrosine nitration include mitochondrial superoxide dismutase [58,59] and several enzymes involved in oxidative energy metabolism, including aconitase [60], glutamate dehydrogenase [46],  $\alpha$ -ketoglutarate dehydrogenase [61], and PDHC [46]. Because PDHC and  $\alpha$ -

ketoglutarate dehydrogenase complex share similar reaction mechanisms and an identical polypeptide subunit (E3), it is not surprising that they are both sensitive to enzyme inhibition by tyrosine nitration. However, Park et al. showed that *in vitro* nitration of tyrosines present in the  $\alpha$ -ketoglutarate dehydrogenase complex was associated with loss of immunoreactivity for the E1 and E2 subunits [61], whereas we observed no loss of immunoreactivity for subunits of SIN-1-treated PDHC (Fig. 4B). Nevertheless, hippocampal PDHC immunoreactivity is lost during hyperoxic reperfusion, coincident with an increase in 3-nitrotyrosine immunoreactivity [14]. Loss of immunoreactivity *in vivo* could, however, be due to degradation by proteases, e.g., the mitochondrial Lon protease, which targets oxidatively modified proteins [62].

The inactivation of PDHC by peroxynitrite, *in vitro*, is primarily due to tyrosine nitration, although the small protection afforded by DTT indicates that S-nitrosation also contributes to enzyme inhibition (Fig. 6). Previously, Bogaert et al. reported that PDHC enzyme activity is inhibited by the hydroxyl radical [5]. This study establishes that PDHC activity is also impaired in the presence of  $ONOO^-$ , another species strongly implicated in oxidative tissue injury [63]. The fact that PDHC enzyme activity can be inhibited by both the hydroxyl radical and peroxynitrite poses the question of which species is responsible for inhibition of this enzyme in the hippocampus during reperfusion. Although the results of our study do not answer this question, the finding that PDHC subunit immunoreactivity is elevated in anti-nitrotyrosine-immunoprecipitated hippocampal proteins suggests that tyrosine nitration contributes to enzyme inhibition *in vivo*. Although tyrosine nitration of PDHC may account for postischemic alterations in metabolism, parallel changes resulting from the effects of peroxynitrite on other cellular components could also contribute to inhibition of cerebral energy metabolism. For example, oxidative stress can damage other proteins, including TCA cycle enzymes [61,64]. Moreover, electron transport chain Complexes I and II are also very sensitive to inhibition caused by reactive oxygen and nitrogen species, including peroxynitrite [65]. The observation that the pyridine nucleotide (NAD(P)H) redox state is hyperoxidized during the first hour of reperfusion after global cerebral ischemia in rats suggests that a reaction before the electron transport chain is the limiting factor in postischemic oxidative energy metabolism [66]. Experiments are in progress to determine if the PDHC reaction is indeed the rate-limiting step.

The molecular mechanisms by which PDHC enzymatic activity is inhibited by peroxynitrite are at this juncture unknown. Whereas many enzymes are subject to inhibition by peroxynitrite, the contribution of tyrosine nitration, S-nitrosation, and other mechanisms to inactivation varies considerably [67–70]. DTT only partially protected against PDHC inhibition, suggesting that tyrosine nitration is the primary mechanism of inactivation. Another possible target is the lipoamide residue located on the E2 subunit of PDHC.

The E2 (dihydrolipoyl transacetylase) subunit may be particularly susceptible to inactivation as the immunoreactivity of this protein is high in anti-nitrotyrosine immunoprecipitates from the hippocampi of hyperoxic resuscitated animals (Fig. 3). Moreover, the E2 subunit of the PDHC was also identified as a target for nitration by Elfering et al. when mitochondria were incubated under conditions of enhanced endogenous nitric oxide production [46]. PDHC is composed of multiple subunits with many different potential targets of peroxynitrite, indicating that several mechanisms of inactivation may be involved.

As we previously demonstrated for inhibition of PDHC by the hydroxyl radical, sensitivity to inhibition by peroxynitrite is modulated by the presence of certain enzyme substrates and cofactors. The protection by pyruvate could be due to either its effects on protein conformation and amino acid availability for oxidation or a direct antioxidant effect of pyruvate that can both scavenge superoxide and react directly with peroxynitrite [71]. Protection afforded by coenzyme A is less likely to be due to antioxidant effects. The relatively low levels of pyruvate and coenzyme A and other PDHC substrates during reperfusion after cerebral ischemia could therefore exacerbate oxidative enzyme inhibition.

Current standards of emergent care after cardiac arrest encourage the use of 100% oxygen during resuscitation. This practice has been questioned by results obtained with animal models demonstrating improved neurologic outcome using postischemic ventilation with O<sub>2</sub> concentrations as low as 21% [15,16,72]. Our present findings demonstrating reduced hippocampal protein tyrosine nitration and retention of PDHC enzyme activity with normoxic resuscitation support the need for additional preclinical and clinical studies to resolve this issue. The concept that hyperoxia worsens oxidative tissue damage and neurologic outcome after acute brain injury may not, however, apply to other forms of injury, e.g., stroke and trauma, as evidence suggests that hyperoxia can under some circumstances be beneficial [73–77]. The time during which the brain is exposed to high O<sub>2</sub> can also determine whether hyperoxia is helpful or detrimental. We found that when dogs were exposed to hyperbaric 100% O<sub>2</sub> 1–2 h after cardiac arrest and resuscitation, both histologic and neurologic outcome were better than in animals maintained under normobaric and normoxic conditions [18]. Abnormally high brain O<sub>2</sub> levels may therefore be toxic primarily during the first 30–60 min after global cerebral ischemia, when abnormal intracellular conditions, e.g., high Ca<sup>2+</sup> and low pH, promote the production of reactive O<sub>2</sub> and N<sub>2</sub> species. Later, high tissue O<sub>2</sub> could promote recovery, particularly if brain oxygenation rather than the activity of metabolic enzymes limits cerebral energy metabolism.

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